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14. ABSTRACT The ongoing study addresses the critical clinical problem of posttraumatic joint stiffness, a pathology that reduces the range of motion (ROM) of injured joints and contributes to the development of osteoarthritis. The fundamental hypothesis that drives the current study is that pathological fibrotic response of injured joint tissues may be limited by targeting the formation of collagen fibrils, a main component of the fibrotic mass. Key preliminary data indicate the following: (i) in comparison to the non-treated control, deposition of newly-formed collagen fibrils in posterior capsules from injured knees of rabbits treated with the anti-fibrotic antibody is reduced significantly, (ii) in comparison to the non-treated control, the correct collagen III/collagen I ratio in posterior capsules from injured knees of rabbits treated with anti-fibrotic antibody is maintained, (iii) in comparison to the non-treated control, the ROM of injured knees of rabbits treated with anti-fibrotic antibody is greater. Ongoing studies with additional groups of animals will determine the statistical significance of the differences observed in the measured parameters. Completion of these experiments will define the utility of the anti-collagen I antibody to block excessive fibrosis associated with joint injury.					
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INTRODUCTION:

This annual report presents a summary of research activities during the first year of funding of a study entitled: “Prevention of the Post-Traumatic Fibrotic Response in Joints”. No changes were made to the original plan presented in the initial statement of work. The current study was awarded through the Translational Research Partnership Award mechanism to test a new approach to reducing trauma-associated stiffness of joints. The presented summary is a result of collaborative work done by a team of basic researchers and a group of orthopaedic surgeons. The ongoing study addresses the critical clinical problem of posttraumatic joint stiffness, a pathology that reduces the range of motion (ROM) of injured joints and contributes to the development of osteoarthritis. This clinical problem is highly relevant not only to military personnel, but also to the civilian population, thus its medical significance is high.

The fundamental hypothesis that drives the current study is that pathological fibrotic response of injured joint tissues may be limited by targeting the formation of collagen fibrils, a main component of the fibrotic mass. To test this hypothesis, we employ an engineered monoclonal antibody that specifically targets collagen I molecules. By binding to the collagen molecules, the antibody prevents their self-assembly to fibrils, thereby reducing the growth of fibrotic tissue. We expect that such reduction will improve the ROM of the injured joint after the healing process is complete and will shorten the time of full recovery. The current study utilizes a clinically-relevant model of post-traumatic joint stiffness. Specifically, this model employs rabbits which undergo a complex surgical procedure to cause trauma to the knee joint. During two months of knee immobilization maintained with the use of a steel pin, the rabbits are treated with the anti-fibrotic antibody administered directly to the knee cavity via a subcutaneous pump. Following the second surgery to remove the pin, the rabbits are allowed to recover for two weeks. After that time, the rabbits are sacrificed, and the collected knees are then analyzed histologically, biochemically, and mechanically. During our first year of this project, we developed a complex surgical procedure and established a method for producing adequate amounts of the therapeutic antibody needed for this study. Moreover, we processed a number of rabbits and obtained key preliminary data. These data indicate the following: (i) the employed rabbit model represents post-traumatic joint stiffness well, (ii) in comparison to the non-treated control, deposition of newly-formed collagen fibrils in posterior capsules from injured knees of rabbits treated with the anti-fibrotic antibody is reduced significantly, (iii) in comparison to the non-treated control, the correct collagen III/collagen I ratio in posterior capsules from injured knees of rabbits treated with anti-fibrotic antibody is maintained, (iv) in comparison to the non-treated control, the ROM of injured knees of rabbits treated with anti-fibrotic antibody is greater. Ongoing studies with additional animals will determine the statistical significance of the differences observed in the measured parameters. Moreover, we will introduce the critical control groups: (i) treated with an anti-inflammatory agent and (ii) treated with the inactive form of antibody. Completion of these experiments will determine the utility of the anti-collagen I antibody to block excessive fibrosis associated with joint injury.

KEYWORDS:

Post-traumatic joint stiffness, anti-fibrotic therapy, collagen, therapeutic antibody, fibrosis, knee joint, animal model, range of motion.

OVERALL PROJECT SUMMARY:

Key participants:

This document offers a comprehensive report of research activities carried out by involved PIs:

Andrzej Fertala, Ph.D., Initiating PI -- Dr. Fertala is an expert in the area of the structure and function of extracellular fibrous proteins with a special focus on collagenous proteins in health and disease. He provides critical input in the areas relevant to the effects of the proposed approach on the structure and functions of

collagenous matrices formed in the presence of the tested molecules. Dr. Fertala oversees and coordinates the entire project and closely interacts with the other PIs and key members of the team.

Joseph Abboud, M.D. Partnering PI -- Dr. Abboud practices as a shoulder and elbow surgeon with a clinical emphasis on fracture care and trauma. His scientific interest includes post-traumatic contractures around the elbow. Dr. Abboud has conducted extensive basic science research with an emphasis on the biomechanical aspects of tendon healing and scar formation in the extremities using various animal injury models. Dr. J. Abboud performs animal surgeries. As a PI, due to his expertise in the field of orthopaedic mechanics, he is also critical for interpreting biomechanical tests of the studied joints. Dr. Abboud also participates in the intellectual process of conceptual design and critical analysis of the results of the performed studies.

Pedro Beredjiklian, M.D., Partnering PI -- Dr. Beredjiklian has a strong track record of basic research in examining the molecular basis of scar tissue and contracture formation after injury and surgery. Dr. Beredjiklian, a practicing orthopaedic surgeon, has a unique perspective on the proposed study. His role is complementary to that of Dr. Abboud, as the number of animals proposed here requires the involvement of two surgeons. In his PI role, Dr. Beredjiklian is responsible for the interpretation of clinically-relevant biological, histological, and morphological results of the analyzed joints.

The clinical observations of both surgeons are discussed with other participating surgeons and with the basic research investigators. Their feedback is critical for modulating initial experimental protocols to circumvent any problems that may occur during the course of the study. Similarly, feedback from the basic research level allows clinical partners to implement changes to the animal-based studies. In addition to the purely technical aspects of the project, broader intellectual involvement of the PIs is key for formulating new concepts and directions for the planned study. Such reciprocal communication is critical for moving the set scientific goals forward and for executing the planned study most productively, thereby ensuring a rapid transition of the proposed approaches from bench to bed. The flow of information is achieved via frequent communication among participating members.

Research activities:

In the first year of funding, no changes were introduced to the original plan of this study. All activities associated with our research activities paralleled those described in the initial SOW.

In the early phase of this project, we focused on developing key techniques needed to successfully execute this current study. Our research activities followed the path defined in specific aims and major tasks:

Specific Aim 1: “To block the fibrotic process after joint injury in a rabbit-based model”

Major Task 1: Production and purification of therapeutic antibodies.

Antibody expression system -- The constant availability of the anti-collagen I antibody is critical for reaching goals set for this project. This recombinant antibody is expressed in CHO cells in the form of IgG containing the light κ chain [1]. To produce the antibody in the amounts needed for this study, we employed a cell culture bioreactor, a specialized device that supports high-density cell cultures (Fig. 1).

Cell preparation – Seeding a bioreactor requires a relatively large number of cells able to grow in serum-free conditions. Moreover, these cells should be able to thrive and proliferate in a suspended form until they are ready for seeding in a bioreactor vessel. To achieve this, we culture CHO cells that express the anti-collagen I antibody in spinner flasks in which the serum content is gradually reduced from 10% to 0% over about one month of culture. Subsequently, these cells are expanded in suspension to reach a specific number needed for seeding a bioreactor.

Bioreactor culture – A bioreactor vessel is seeded with CHO cells “trained” to grow in serum-free conditions (Fig. 1). The cells are maintained in a serum-free, protein-free, chemically-defined CD CHO™ medium (Life Technologies).

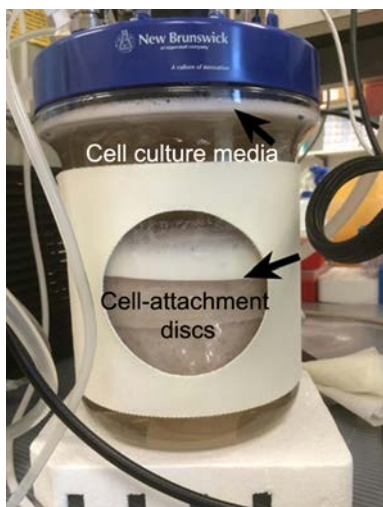


Figure 1. A 3.5-L bioreactor vessel to culture CHO cells that produce the anti-collagen I antibody. Cell culture media and porous discs that capture cultured cells are indicated.

Each day the performance of cell culture is monitored by observing the consumption of O_2 , and glucose. Other parameters such as pH, temperature, and delivery of CO_2 are also recorded. The cell culture medium is exchanged every 2-3 days of culture: 3 L of the conditioned media containing the secreted antibody is collected into a sterile container placed in a refrigerator while the same volume of fresh medium is added to a vessel. This routine is continued until the culture ceases its function, as evident by low glucose and O_2 consumption. Typically, a culture performed well for 4-5 weeks generating about 40 L of conditioned media.

As cell culture media used in bioreactor-based cell cultures require low concentrations of non-ionic surfactants to protect cells from the shear forces imposed by circulating media, foaming is a common problem. To reduce the foaming issue, we employed FoamAway™ (Life Technologies), the anti-foaming agent added periodically into an active culture.

Purification of antibodies – Our initial plan to purify the anti-collagen I antibody by salt precipitation and ion-exchange chromatography has failed. Although this method works well for purification of the anti-collagen I antibody derived from regular CHO cell cultures, it could not be applied for the bioreactor-derived material. The reason for this was the presence of a surfactant added to the CD CHO™ medium. Despite testing various options, it was not possible to completely eliminate this material whose presence interfered with the antibody purification process.

The problem of the presence of the surfactant was eliminated by employing affinity chromatography in which we employed Protein L agarose (GenScript). This resin is highly specific toward binding the κ chain, an element of the anti-collagen I antibody applied in our study. Thus, upon collecting the conditioned media, the whole media batch was filtered to remove the cell debris. Subsequently, the medium was passed through a Protein L column to capture the antibody. Next, the column was washed extensively with phosphate buffered saline (PBS) to remove traces of non-bound contaminants, and then the antibody was eluted with a glycine buffer, pH=3.0. Following the concentration of isolated antibody, its purity was assayed by polyacrylamide gel electrophoresis. Moreover, we also employed size exclusion chromatography to analyze the potential aggregation of the purified antibody [1].

As indicated in Fig. 2A, B, the purified antibody was homogenous and did not contain any contaminating proteins. Moreover, size exclusion chromatography has determined that the antibody was present in non-aggregated monomeric form. As indicated in Fig. 2B, the elution profile shows only one major peak during which the elution volume was consistent with a 150-kDa protein. Such a molecular mass is consistent with the mass of the recombinant anti-collagen I antibody employed here and with native IgG (Fig. 2B).

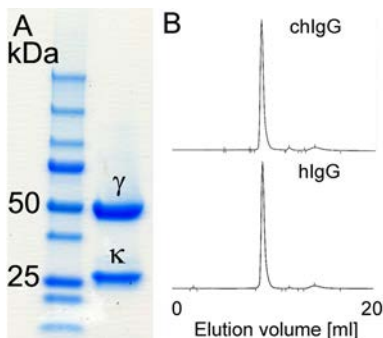


Figure 2. Electrophoretic (A) and chromatographic (B) assays of purified chimeric anti-collagen I antibody (chIgG); a chromatographic profile of native human IgG (hIgG) is also presented. In A, markers of molecular masses are also presented; electrophoretic migration of the heavy γ and the light κ chains of chIgG is presented.

Testing the activity of purified antibody – Purified and concentrated antibody was dialyzed to PBS and then sterilized by filtration through a 0.2- μ m filter.

Subsequently, the activity of the antibody was tested against its natural target, namely the C-terminal telopeptide of collagen I, as described [1, 2]. In brief, this test is based on Western blot where a nitrocellulose membrane-bound target is detected with purified anti-collagen I antibody. Figure 3 depicts a typical positive result of a Western blot assay demonstrating the anti-collagen I antibody/collagen I binding.



Figure 3. A Western blot assay to test the binding of the anti-collagen I antibody to the $\alpha 2(I)$ chain of procollagen I. Positive signals indicate the expected binding interaction. Two bands apparent in a blot represent intact and partially processed procollagen I $\alpha 2$ chain.

Testing the long-term stability and activity of the anti-collagen I antibody - As described in the original application, the antibody is delivered to the site of injury via a refillable pump implanted subcutaneously during surgery. In brief, the pump's reservoir is filled to a maximum capacity of 0.9 ml. The antibody is then constantly delivered at a rate that empties the reservoir after 4 weeks of operation. To continue the delivery of the therapeutic antibody, the reservoir is refilled via a subcutaneous port so that the delivery of the antibody continues for the next 4 weeks, for a total 8 weeks. Because of this delivery regime, it has been necessary to determine the stability and the activity of the anti-collagen I antibody kept in a pump for 4 weeks at 37°C. To do this, an operating pump filled with the tested antibody was maintained for 4 weeks in a cell culture incubator. After 4 weeks of constant incubation, the structural integrity and activity were tested as described above. As indicated in Fig. 4, the antibody remained intact (Fig. 4A) and maintained its activity (Fig. 4B). Moreover, in one experiment, an aliquot of the antibody was retrieved from a pump collected from a rabbit after 8 weeks of operation. As demonstrated in Fig. 4C, the antibody was intact, thereby indicating its great stability in the experimental environment of the rabbit model. Together, these data offer evidence for the stability and activity of the antibody over the experimental period.

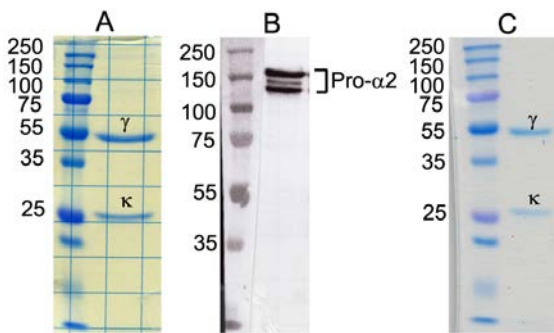


Figure 4. Analyses of the structural integrity and the activity of the anti-collagen I antibody kept at 37°C for an extended period. A, An electrophoretic assay of the antibody stored constantly at 37°C for 5 weeks. B, Detection of procollagen I (Pro- $\alpha 2$) of antibody shown in A indicates its activity after continuous, 5-week incubation at 37°C. C, An electrophoretic assay of antibody retrieved from a pump implanted subcutaneously in a rabbit for 8 weeks. Note that the structure of the antibody remains intact. Markers of molecular masses and the γ and κ chains of the anti-collagen antibody are indicated.

Blocking the activity of the anti-collagen I antibody- As proposed in the original application, in addition to a group receiving the active form of the anti-collagen I antibody, a control group receiving the inactive form of this antibody will be also included. We proposed to inactivate the antibody either via thermal denaturation or by modification of the antigen-binding sites. Our recent attempts to thermally denature the antibody caused its aggregation and subsequent precipitation, thereby rendering such a method of inactivation unsuitable. Next, considering the relatively high content of the lysine residues present in the tested antibody (unpublished data), we blocked the ϵ -amino groups of the side chains of these residues by employing N-hydroxysuccinimide (NHS)-based chemistry (Pierce, Thermo Scientific), a compound that reacts with the primary amines in neutral pH. Subsequently, the modified anti-collagen I antibody was tested for its reactivity with collagen I via Western blot, as described above. As depicted in Fig. 5, in contrast to the active form of the anti-collagen I antibody

(Fig. 5A), the procollagen I target was not detected by the blocked form of antibody (Fig. 5B), thereby indicating its inactivity. Consequently, the inactive form will be employed in a control group of rabbits, according to the initial plan outlined in the original application.

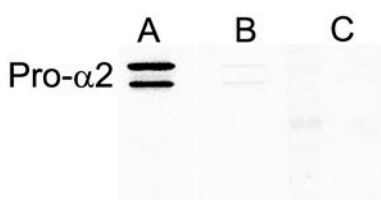


Figure 5. Analysis of the effectiveness of blocking the activity of the anti-collagen I antibody. A, Detection of procollagen I-target (Pro- α 2) with control, active form of the anti-collagen I antibody. B, Lack of the procollagen I-specific signal indicates the effective blocking of the anti-collagen I antibody. C, Control Western blot done with the use of native human IgG with no specificity against collagen I.

Major Task 2: Testing procedures for generating a rabbit-based model of joint stiffness.

Developing experimental model - The rabbit-based model employed in this study offers a biologically-relevant environment to test the utility of the anti-collagen I antibody to block fibrosis associated with injury to a joint [3-5]. Applying this model requires skilled surgeons to carry out complex procedures to induce post-traumatic joint stiffness (Fig. 6).

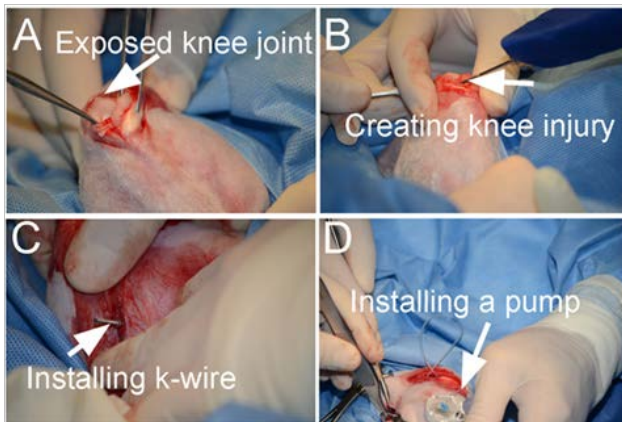


Figure 6. Selected elements of surgery performed to induce post-traumatic joint stiffness in experimental rabbits. A: exposing the knee joint, B: creating injury in the knee joint, C: installation of a k-wire to immobilize the knee joint in a flexed position, D: installation of a refillable pump for continuous intra-articular delivery of the tested compound.

In brief, these procedures include causing destabilization of the knee joint, inducing intraarticular bleeding, installing a k-wire to immobilize the knee joint, and implanting a pump to deliver the therapeutic antibody. Moreover, after 8 weeks of immobilization, the second surgery to remove a k-wire is performed. Consistent with the guiding principle of the Translational Research Partnership Award that encourages multi-institutional, multidisciplinary research partnerships among investigators, these animal-based studies are carried out by a group of orthopaedic surgeons led by Drs. Beredjikian and Abboud. Indeed, during the first year of the study, we successfully enrolled and actively engaged a group of surgeons that include Drs. Wang, Barlow, Namdari, Arnold, and more recently, Dr. Rivlin (pending IACUC approval).

At the initial phase of implementing the animal model, a few unexpected technical problems had to be solved. The first problem was a result of employing k-wires that were too short to form a correct hook needed to keep it firmly positioned over a femur (Fig. 7). As a result, two k-wires slipped from femora where they were positioned during a surgical operation. To prevent this problem, we designed longer k-wires, so now the critical hooks are readily formed with the use of a k-wire bender (Fig. 7). Since implementing this change, no k-wire slippage has occurred.

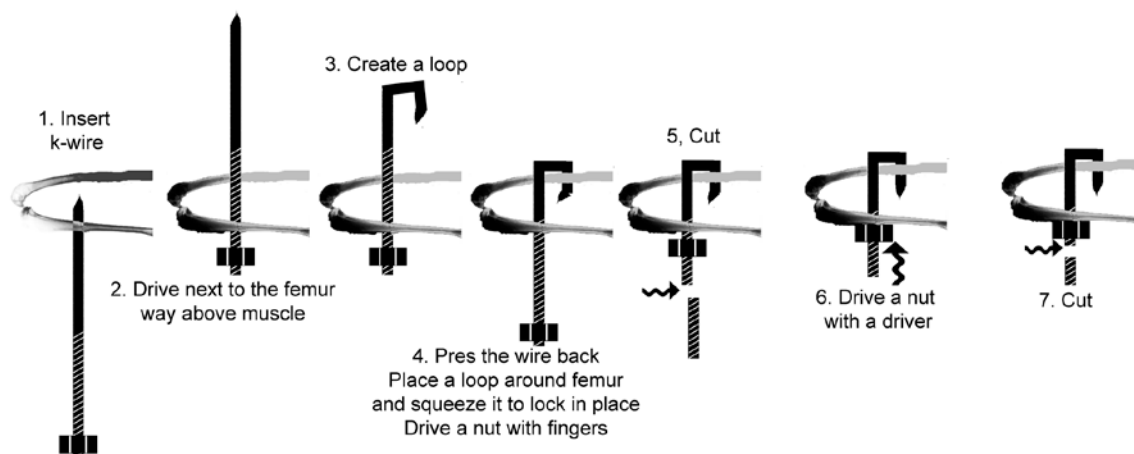


Figure 7. A schematic depicting a technique to immobilize the rabbits' knees.

The second technical problem was associated with immobilization of a silicon tube that delivers the antibody from a pump to the injured joint. As such a tube runs through a femoral condyle, it has been difficult to secure it with the sutures. To eliminate this problem, we designed a tube whose one end includes a retention bead. Such a bead, whose diameter is slightly larger than that of the actual tube, prevents the tube from sliding out from its position within the knee cavity (Fig. 8). At present, we are confident that all elements of our rabbit-based experimental model are optimized, so we do not expect any technical problems to interfere with our study.



Figure 8. Processing of a rabbit's leg to harvest the anterior and posterior knee capsule. The insert indicates a retention bead (arrow) that keeps a tube used to deliver the anti-collagen I antibody in the correct position.

Testing the path of antibody delivery - A contrast agent was employed to ensure the continuous delivery of the therapeutic antibody to the injured knee through the entire 8-week delivery period. In brief, eight weeks after implanting the pump, when the pump's reservoir was almost empty, the reservoir was filled with an approved contrast agent. Following a few hours of operation, an X-ray was taken to observe the flow of contrast.

Figure 9 depicts the continuous flow path from the pump to the knee cavity, thereby demonstrating the reliability of the employed antibody-delivery system.

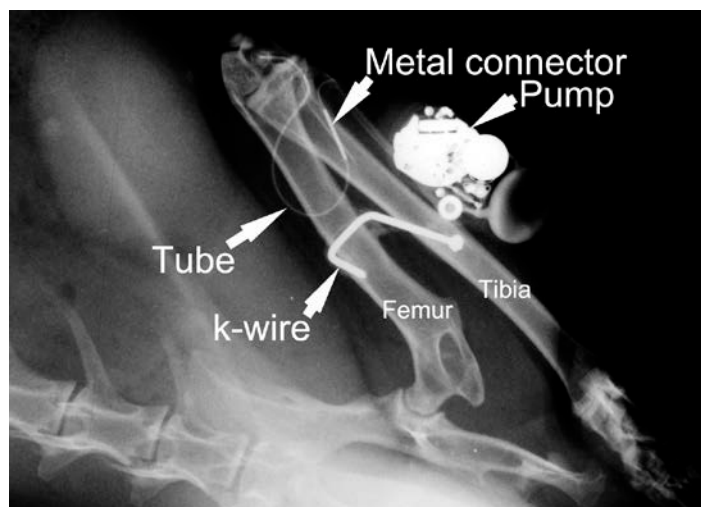


Figure 9. An illustration of continuous flow path from the pump to the knee cavity. The depicted flow is visualized by employing a contrast agent injected to the pump.

Harvesting and processing rabbits' legs - Ten weeks after the initial surgery, the rabbits are sacrificed, and then the injured leg and a contralateral uninjured leg are harvested. The legs are thoroughly examined to observe any atypical features that might develop after surgery within their musculoskeletal tissues. Subsequently, the legs are then processed for mechanical, histological, and biochemical assays.

Mechanical tests – The ultimate goal of the ongoing study is to test the efficacy of the anti-collagen I antibody to block fibrosis, a major factor causing post-traumatic joint stiffness. We postulate that a key indication of the antifibrotic action of the anti-collagen I antibody will be increased ROM of antibody-treated joints vs. non-treated controls. To facilitate the measurement of the ROM, we acquired a custom-made device (TestResources Inc., Shakopee, MN) purchased with intramural funds (Fig. 10).

For the mechanical tests, the collected legs are processed to remove most of the muscle tissue, leaving the tissues surrounding the knee intact (Fig. 10). Subsequently, the tibia and femur of the analyzed leg are cut at the ankle and hip joints, respectively. Next, the ends of bones are embedded in a resin to facilitate a secure clamping in the instrument (Fig. 10). Finally, the maximum angle at the force of 0.2 Nm is measured and recorded (Fig. 10 & 11). The measured angles for injured legs are presented as a percent of the angle measured for the contralateral uninjured leg.

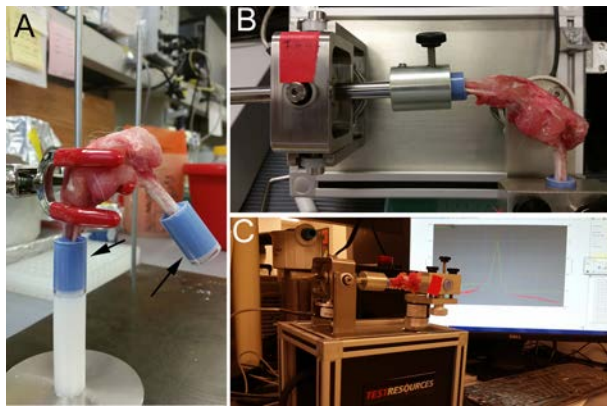


Figure 10. Mechanical assays of the rabbits' legs. A, Embedding (arrows) of bones facilitates their secure clamping in a device (B, C) used to measure joint contracture angles.

Our measurements to date indicate that the maximal angles for the antibody-treated knees are greater than those measured for non-treated injured controls, thereby suggesting improvement of the ROM. In particular, our initial mechanical tests indicate increased extension of the antibody-treated knees in comparison to the control. Specifically, our current data indicate that the average extension angle of the injured, non-treated knees was 24.3% of the non-injured contralateral control, while the corresponding value for the injured, antibody-treated knees increased to 37.3%. Note that these data are just preliminary and, at present, cannot be considered statistically valid. Ongoing and planned studies with additional rabbits and testing control groups such as those treated with the inactive form of the anti-collagen I antibody will allow the formulation of final conclusions.

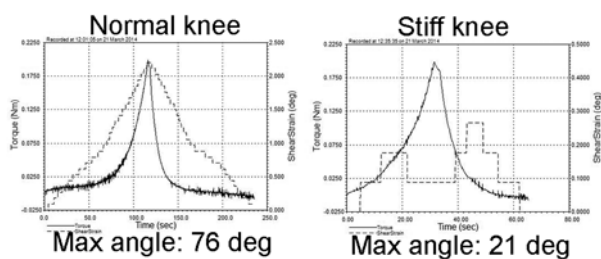


Figure 11. Representative results of the measurement of the stiffness of the knee joints of experimental rabbits. Each curve represents the behavior of the analyzed knee joint during extension and flexion cycles.

Assays of fibrotic knee capsules- Following mechanical tests, the legs are processed to dissect the posterior and anterior knee capsule (Fig. 8). At present, however, our main focus is on the posterior capsules, as it has been demonstrated that their fibrotic changes are the main cause for fibrotic changes and the stiffness of the knee. Following dissection, a capsule is divided to three parts: (i) for quantitative assays of total collagen, (ii) for qualitative assays of collagen, and (iii) for histological assays.

Assays of collagen content - In the studies performed to date, the total collagen content in isolated capsules was analyzed by determining the content of hydroxyproline, a collagen-specific amino acid, in a unit mass of dry tissue [6]. In brief, a portion of a capsule was frozen in liquid nitrogen and then mechanically

pulverized. Subsequently, tissue lipids that interfere with the hydroxyproline assays were extracted with a chloroform/methanol mixture. The organic phase containing lipids was then removed. Next, the samples were dried by lyophilization and their masses were recorded. Afterward, each sample was hydrolyzed in 6 N HCl and the concentration of hydroxyproline was determined according to established methods. Finally, the content of the total collagen/mg of dry mass was calculated.

The results of collagen assays indicate that the content of collagen in posterior knee capsules is relatively high in all analyzed groups. Specifically, total collagen comprises >80% of dry mass to the posterior capsule. Although we observed a slight decrease of the total collagen in the capsules from the injured knees treated with the anti-collagen I antibody, this decrease was not statistically significant when compared to non-treated control (Fig. 12) To explain this result, we analyzed the histology of the fibrotic and healthy capsules (Fig. 14). We observed that the fibrotic one is much thicker in comparison to uninjured control; in fact this difference was already apparent during the macroscopic evaluation of the capsules done at the time of their harvest. Consequently, we conclude that contributing >80% of dry mass collagen is the main component of both fibrotic and healthy capsules.

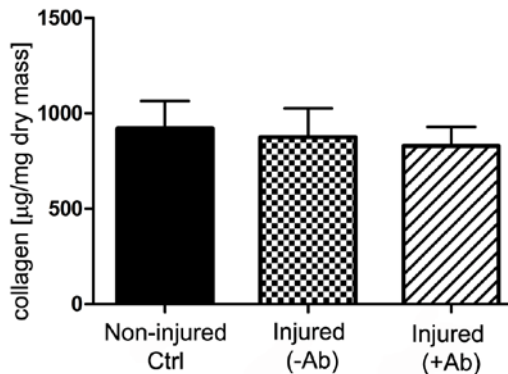


Figure 12. Assays of the total collagen content per unit of dry mass of posterior capsules isolated from uninjured knees (Ctrl), injured not-treated with the anti-collagen I antibody (-Ab) and injured treated with the antibody (+Ab).

Qualitative assays of collagen - As collagen is the main building material of capsules, we also analyzed the composition of a pool of collagen proteins. In particular, we focused on two dominant collagen types, namely collagen I and collagen III. We consider that observing changes in the collagen composition is important for establishing parameters that characterize the fibrotic process and repair of the

damaged joint capsule. For these assays, collagen was extracted from the capsules with the use of pepsin. Extracted collagen was analyzed by polyacrylamide gel electrophoresis whose representative result is depicted in Fig. 13. Assays to determine the relative contents of collagen I and collagen III are currently ongoing with the use of collagen I-specific and collagen III-specific antibodies.

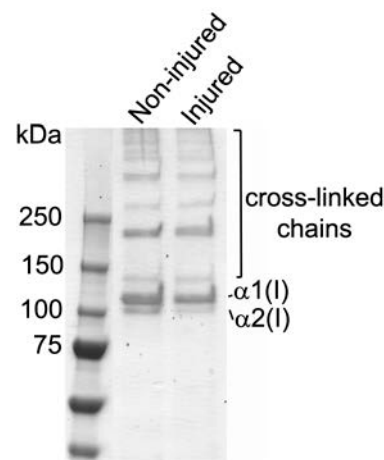


Figure 13. An electrophoretic separation of collagen fractions extracted from analyzed capsules. A Coomassie blue-stained gel collagen demonstrates the presence of monomeric collagen molecules and various fractions of cross-linked molecules.

Histological assays - As indicated above, portions of the knee capsules were processed for histology. First, the samples were stained with hematoxylin/eosin for general morphology and cellularity. As depicted in Fig. 14A, the typical morphology of a normal capsule is a relatively thin structure of connective tissue surrounded by fatty deposits. In contrast, the fibrotic posterior capsule becomes thick due to increased deposition of collagenous tissue (Fig. 14B).

Immunohistology to detect markers of fibrosis - The existence of the fibrotic process in injured capsules was confirmed by detecting selected markers. Figure

14 shows the presence of fibrotic cells that express connective tissue growth factor (CTGF), an element of fibrotic response that promotes the biosynthesis of elements of fibrotic tissues, including collagens (Fig. 14D).

We have also analyzed changes in the expression of elements needed for the production of collagenous proteins. As indicated in Fig. 15, the expression of heat shock protein 47 (HSP-47), a chaperone critical in collagen folding, has increased in cells present in fibrotic capsule. In contrast, in cells present in the healthy capsule, the amount of this protein was markedly lower (Fig. 15). Moreover, we also demonstrated the increase of α -smooth muscle actin, yet another fibrotic marker present in the capsules of injured knees (not shown).

The above assays of the selected markers confirm the occurrence of fibrosis at the cellular level, thereby further rendering the selected animal model biologically relevant.

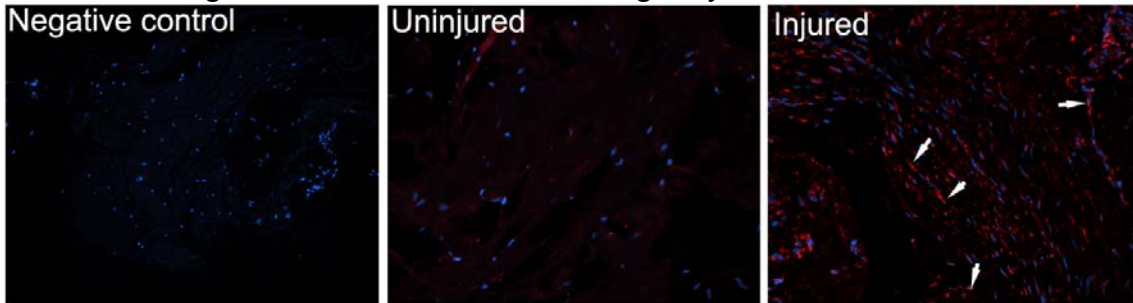


Figure 15. Immunostaining of posterior capsules for the presence of HSP-47. As indicated by the arrows, HSP-47 is abundant in cells present in the capsules isolated from injured knees. In contrast, HSP-47-positive staining is not apparent in cells present in uninjured tissue. The negative control in which no primary anti-HSP-47 antibody was used is also indicated.

Collagen-specific staining of fibrotic capsules - The inhibitory potential of the anti-collagen I antibody is also evaluated directly at the level of collagen fibril present in the posterior capsules. In particular, collagen fibrils are stained with Sirius red, a dye that specifically binds to collagen fibrils (Fig. 14E). Collagen fibrils stained with this dye are characterized by birefringence when observed in the polarized light. It has been recognized that well-established mature collagen fibrils observed via a polarizing microscope have a distinct red color, while newly formed fibrils appear as green-colored filaments [7]. Our preliminary results on the collagen fibrils present in posterior capsules indicate that, in injured rabbits not treated with the anti-collagen antibody, the relative content of the new fibrils formed as a result of the fibrotic processes was high (53.7%). In contrast, in the corresponding group treated with the anti-fibrotic antibody, the relative content of new collagen fibrils was markedly lower (25.8%). The content of thin, novel collagen fibrils in non-injured posterior capsules was 20.6% of the total collagen fibril content (Fig. 14E).

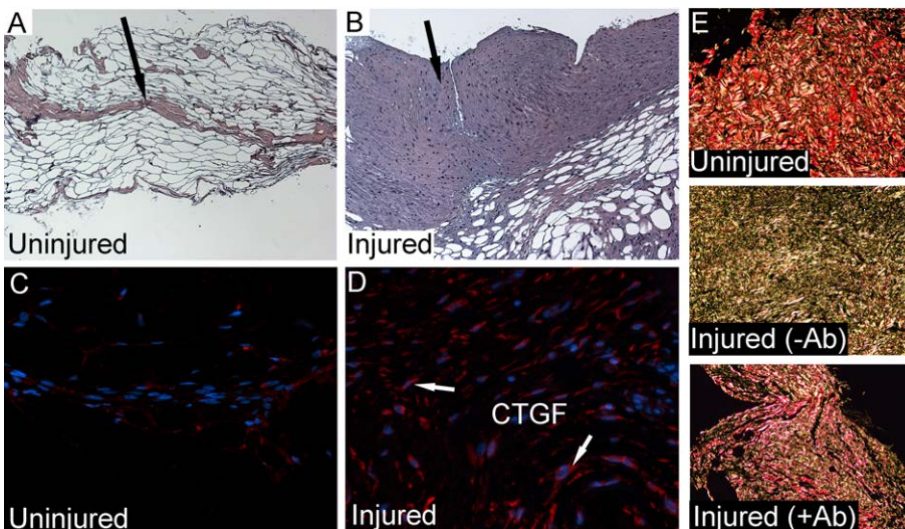


Figure 14. General morphology of the posterior capsule from an uninjured knee (A) and an injured knee (B); arrows indicate the structure of collagen-rich tissue. Immunostaining assays of CTGF indicate a background-level signal in a healthy posterior capsule (C) and markedly elevated presence of CTGF (arrows) in the fibrotic counterpart (D). E, A polarized-light image of the Sirius red-stained fibrils from an uninjured knee and from the injured knees maintained in the absence (-Ab) or the presence of the anti-collagen antibody (+Ab).

(+Ab) of the anti-fibrotic antibody. Note that in the presence of the anti-collagen antibody, the amount of newly-formed fibrils (green) is markedly reduced.

Analyzing the influence of the anti-collagen I antibody in the surface of articular cartilage - Since the anti-collagen I antibody is continuously delivered for 8 weeks into the injured knees, we tested whether it affects the morphology of the articular cartilage of treated joints. Figure 16 indicates that the surface of articular cartilage isolated from a rabbit treated with the anti-collagen I antibody had normal morphology characterized by a smooth surface, uniform distribution of chondrocytes, and abundant proteoglycans.

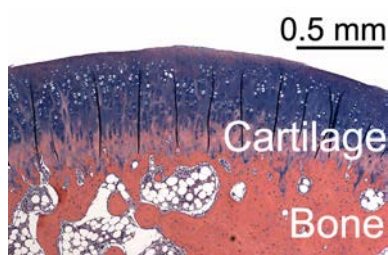


Figure 16. Hematoxylin and Alcian blue staining of cartilage isolated from a rabbit's knee treated with the anti-collagen I antibody for 8 weeks.

KEY RESEARCH ACCOMPLISHMENTS:

- Identification of key elements of the fibrotic process occurring in the capsule of the injured knee: (i) the fibrotic process involves cell activation as evidenced by the presence of α -smooth muscle actin and CTGF, (ii) the fibrotic process involves activation of intracellular elements that control collagen synthesis as evidenced by the increase in HSP-47.
- Preliminary data indicate the beneficial effects of the anti-collagen I antibody on reducing fibrotic response associated with knee injury.

CONCLUSION:

We have demonstrated that the employed animal model offers a biologically-relevant environment to study post-traumatic joint stiffness. The results of our study to date suggest that applying the anti-collagen I antibody to the injured knee reduces the amount of newly deposited collagen fibrils formed in response to the knee injury. Moreover, preliminary mechanical assays of the knee motion provide initial evidence that the ROM of antibody-treated knees may improve as a result of such treatment. Currently, however, our data are incomplete and have to be supplemented by results from a group of rabbits treated with the inactive form of the anti-collagen I antibody, control rabbits treated with an anti-inflammatory agent, and additional rabbits from groups with longer recovery periods, as described in the original proposal. These groups are continuously generated, maintained, and tested.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

An abstract has been submitted for presentation during the Orthopaedic Research Society conference scheduled for March 2015 (see Appendix):

"Testing the Utility of Engineered Anti-Collagen I Antibody to Limit the Formation of Collagen-Rich Fibrotic Deposits in a Rabbit Model of Posttraumatic Joint Stiffness". Steplewski, Andrzej¹, Fertala, Jolanta¹, Barlow, Jonathan^{1,2}, Beredjiklian, Pedro^{1,2}, Abboud, Joseph^{1,2}, Wang, Mark^{1,2}, Namdari, Surena^{1,2}, Arnold, William^{1,2}, Kostas, James¹, Cheryl Hou¹, and Fertala, Andrzej¹

¹Department of Orthopaedic Surgery, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, United States. ²Rothman Institute of Orthopaedics, Thomas Jefferson University Hospital, Philadelphia, PA, United States.

INVENTIONS, PATENTS AND LICENSES:

None.

REPORTABLE OUTCOMES:

Research tool: An animal model to test the constant delivery of a therapeutic agent to the injured joint.

OTHER ACHIEVEMENTS:

This study provided a valuable training opportunity for a group of orthopaedic surgeons who are interested in translational approaches aiming at the development of a novel treatment. The following surgeons were attracted and are actively involved in this study: Drs. Wang, Barlow, Namdari, Arnold, and more recently, Dr. Rivlin (pending IACUC approval).

Encouraged by the results of this study, an intramural proposal (pending review) entitled "Inhibition of Formation of Abdominopelvic Adhesions" was recently submitted by a group of general surgeons. Moreover, discussions on a potential grant proposal were initiated with eye doctors interested in the inhibition of fibrosis associated with glaucoma surgery.

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APPENDICES:

An abstract submitted for the ORS conference.

Testing the Utility of Engineered Anti-Collagen I Antibody to Limit the Formation of Collagen-Rich Fibrotic Deposits in a Rabbit Model of Posttraumatic Joint Stiffness.

Steplewski, Andrzej¹, Fertala, Jolanta¹, Barlow, Jonathan^{1,2}, Beredjickian, Pedro^{1,2}, Abboud, Joseph^{1,2}, Wang, Mark^{1,2}, Namdari, Surena^{1,2}, Arnold, William^{1,2}, Kostas, James¹, Cheryl Hou¹, and Fertala, Andrzej¹

¹Department of Orthopaedic Surgery, Sidney Kimmel Medical College, Thomas Jefferson University, Philadelphia, PA, United States. ²Rothman Institute of Orthopaedics, Thomas Jefferson University Hospital, Philadelphia, PA, United States.

A significant element of the pathomechanism associated with posttraumatic joint stiffness is fibrosis of joint tissues. Development of fibrosis is a complex process initiated in response to joint injury. In brief, the early process of inflammation attracts circulatory and resident cells, which secrete various growth factors. Consequently, these growth factors stimulate the migration of cells into the injury site and promote their proliferation. As a result, these cells actively produce various elements of the extracellular matrix needed to repair damaged joint tissues. In the fibrotic process, however, the synthesis and assembly of newly formed connective tissue is not balanced with the process of its degradation, thereby leading to the formation of pathological deposits of extracellular material. The main element of this fibrotic material is fibrils formed by the assembly of individual collagen I molecules. The excessive amount of collagen fibrils present in the fibrotic capsule contributes to the reduction of the range of motion (ROM) of the affected joint.

Because current approaches to reduce joint stiffness are not fully successful, our group formulated a hypothesis that collagen fibril formation, a key extracellular step in development of fibrotic deposits, represents an attractive target to reduce posttraumatic joint stiffness. The premise for this hypothesis is rooted in the fact that collagen fibril formation is a process driven by the self-assembly of individual collagen molecules and in the indication that blocking this process would reduce the mass of pathological fibrotic deposits, thereby reducing overall joint stiffness.

To test this hypothesis, we engineered an antibody that specifically recognizes and binds to a key domain of collagen I monomers, thereby preventing their assembly into fibrils. Our preliminary results done *in vitro* and in organotypic models of fibrosis indicated the potential utility of this antibody to limit collagen fibril formation [1, 2]. Here we present the novel preliminary results of our study, carried out in a rabbit-based model of posttraumatic joint injury. In brief, rabbits' knees were injured by employing a standardized technique, as described (Fig. 1) [5]. In each processed rabbit, the contralateral knee served as the uninjured control. Employing a k-wire, the injured leg was immobilized in a flexed position for eight weeks. During this time, the therapeutic antibody was continuously administered to the injury site via a pump installed subcutaneously (Fig. 1). Following eight weeks of immobilization, the k-wires were removed and the rabbits were allowed to move freely for two weeks. Subsequently, the rabbits were sacrificed and processed for mechanical, biochemical, and histological analyses (Fig. 1). First, employing a custom-made device (Fig. 1), the extension torque was applied to a maximum of 0.2 Nm, as described [5]. The extension angles were recorded to describe the degree of contracture of the analyzed knee joints. Next, the posterior knee capsules were dissected and processed for biochemical, histological, and immunohistological assays. The biochemical assays of collected tissues were performed to characterize the total collagen content, its composition, and degree of crosslinking. Moreover, tissue samples were stained with Sirius red and then collagen fibrils were studied with the use of a polarizing microscope to determine their quality and the extent of the fibrotic process in the analyzed samples. Specific immunohistology assays were conducted to characterize selected markers of the fibrotic process.

Mechanical analyses of the injured knees indicate severe contracture of the injured joints in the rabbit model employed here. The fibrotic morphology of the posterior knee capsules (Fig. 2B), and the presence of fibrotic markers such as connective tissue growth factor (CTGF, Fig. 2D), further demonstrated the development of the fibrotic tissue. No fibrotic changes were observed in uninjured contralateral knees (Fig. 2A, C). Polarized-light microscopic assays of collagen fibrils present in the healthy posterior capsules indicate that they comprise mature thick fibrils of a uniform morphology with a red/orange appearance. In contrast, the fibrotic capsules include numerous immature thin fibrils whose appearance is predominantly green (Fig. 2E).

The first-round analyses of the above parameters in a group of rabbits whose injured knees were exposed to the anti-collagen antibody demonstrated the reduction of fibrosis and the consequences of this reduction. Specifically, our initial mechanical tests indicate increased extension of the antibody-treated knees in comparison to the control. Specifically, our current data indicate that the average extension angle of the injured, non-treated knees was 24.3% of the non-injured contralateral control, while the corresponding value for the injured, antibody-treated knees increased to 37.3% ($p < 0.05$). Our preliminary results on the quality and quantity of collagen fibrils present in the posterior capsules indicate that, in the injured rabbits not treated with the anti-collagen antibody, the relative content of the new fibrils formed as a result of the fibrotic processes was high (53.7%). In contrast, in the corresponding group treated with the anti-fibrotic antibody, the relative content of new collagen fibrils was markedly lower (25.8%). The content of thin, novel collagen fibrils in non-injured posterior capsules was 20.6% of the total collagen fibril content (Fig. 2E).

The biochemical assays do not indicate any significant changes in the total collagen content per unit of the dry mass of analyzed posterior capsules, thereby indicating that both non-fibrotic and fibrotic capsules have high collagen content of about 90% of their dry mass. Ongoing studies on the content of collagen fibril-stabilizing cross-links and the contribution of specific collagen types will provide further information on the effects of the anti-collagen antibody on the development of joint fibrosis.

In summary, our preliminary data indicate that the anti-collagen antibody that targets a key collagen I domain reduces the amount of newly-formed collagen fibrils in an injured joint capsule, thereby improving the ROM of an antibody-treated knee.

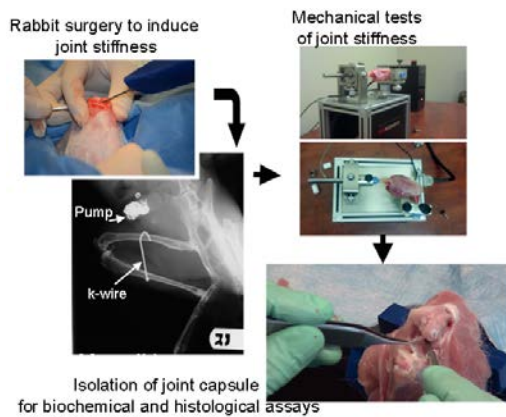


Figure 1. Illustration of critical steps of the described study.

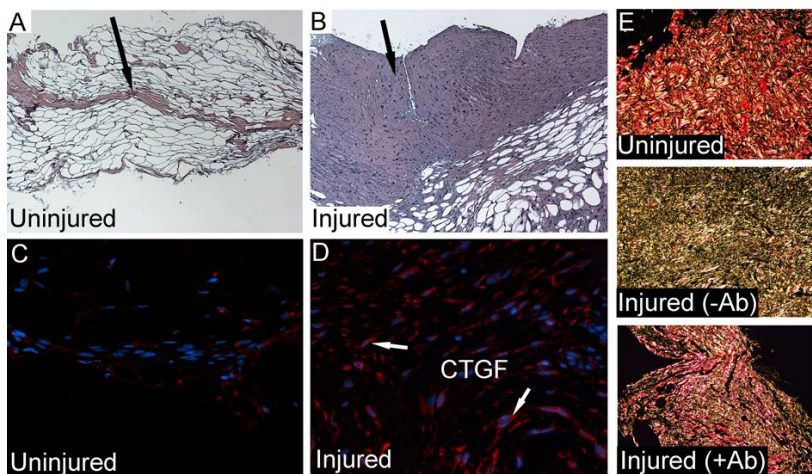


Figure 2. General morphology of the posterior capsule from an uninjured (A) and an injured knee (B); arrows indicate the structure of collagen-rich tissue. Immunostaining assays of CTGF indicate a background-level signal in a healthy posterior capsule (C) and markedly elevated presence of CTGF (arrows) in the fibrotic counterpart (D). E, A polarized-light image of the Sirius red-stained fibrils from an uninjured knee and from the injured knees maintained in the absence (-Ab) or the presence (+Ab) of the anti-fibrotic antibody. Note that in the presence of the anti-collagen antibody, the amount of newly-formed fibrils (green) is markedly reduced.

Significance:

Posttraumatic joint stiffness represents a major medical problem. Because our study aims at developing new approaches to limit this problem, its significance is high.

Acknowledgements:

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